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A KINETIC METHOD FOR THE MEASUREMENT OF ZINC INFLUX *IN VIVO* IN THE RAINBOW TROUT, AND THE EFFECTS OF WATERBORNE CALCIUM ON FLUX RATES

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Summary

Three methods were evaluated to measure rate of influx of Zn into rainbow trout. The first two, disappearance of ^{65}Zn from the water and whole-body counting, overestimated influx when compared with a third method which used a terminal plasma sample to calculate influx. The cause of the overestimate was a short-term adsorption phenomenon to both the experimental apparatus and the exterior of the fish. The third method measured only Zn which entered the fish. This method entailed 'calibration' of cannulated trout by constant infusion of small amounts of radiolabelled Zn. This was analogous to the entry of Zn into fish across the gill. After 24–36 h of infusion, plasma radioactivity reached a steady-state concentration which was a simple linear function of the rate of infusion. This relationship was then used to predict influx from a single terminal plasma sample from uncannulated trout exposed to radiolabelled Zn in the water. Trout acclimated to tapwater ($Ca^{2+} = 2{\cdot}0\,mequiv\,l^{-1}$) and exposed to Zn ($1{\cdot}5–45{\cdot}9\,\mu equiv\,l^{-1}$; $0{\cdot}05–1{\cdot}5\,mg\,l^{-1}$) showed saturable uptake which was apparently first order with no significant linear component. The apparent J_{max} and K_m were $314\,nequiv\,kg^{-1}\,h^{-1}$ and $7{\cdot}3\,\mu equiv\,l^{-1}$ ($0{\cdot}24\,mg\,l^{-1}$), respectively. Acutely raising the waterborne $[Ca^{2+}]$ ($4{\cdot}7$ and $9{\cdot}7\,mequiv\,l^{-1}$) over the same range of [Zn] revealed a competitive type of interaction – little change in J_{max}, with increased K_m. When Ca^{2+} was acutely removed ($0{\cdot}05$ and $1{\cdot}02\,mequiv\,l^{-1}$) by the use of artificial soft water, significant linear influx occurred in addition to the saturable uptake noted at higher $[Ca^{2+}]$, suggesting the opening of a paracellular leak. Calculation of the inhibitor constant for Ca^{2+} yielded a value of $0{\cdot}48\,mequiv\,l^{-1}$. This value is similar to the K_m for Ca^{2+} when it was a transported substrate ($0{\cdot}28 \pm 0{\cdot}07\,mequiv\,l^{-1}$). The true K_m for Zn transport in the absence of Ca^{2+} was $1{\cdot}0\,\mu equiv\,l^{-1}$ ($0{\cdot}06\,mg\,l^{-1}$). These data showed Zn influx to be saturable and strongly dependent upon waterborne $[Ca^{2+}]$, perhaps traversing the gill in a manner similar to Ca^{2+}.

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Introduction

Water can be an important source of Zn to rainbow trout. Below the toxic threshold, the significance of waterborne Zn relative to dietary Zn increases with increasing waterborne concentration (Milner, 1982; Spry *et al.* 1988). We have demonstrated direct transbranchial transport of Zn *in vitro* using an isolated, perfused trout head preparation (Spry & Wood, 1988). This preparation yielded influx rates of about 4–6 nequiv $kg^{-1} h^{-1}$ in hardwater-acclimated trout. These rates are very low compared with those for Na^+ and Cl^-, and even Ca^{2+} (Hõbe *et al.* 1984; Perry & Wood, 1985). They are, however, very close to rates measured for free Cd^{2+} using a similar *in vitro* preparation (Pärt & Svanberg, 1981; Pärt *et al.* 1985).

Although loss of ions across the gill reflects passive movement down electrochemical gradients, there is good evidence that Na^+ and Cl^- influx across the gills of freshwater fish is by active, electroneutral carrier-mediated exchanges against the gradient (see Maetz, 1971; Girard & Payan, 1980; Payan *et al.* 1984). Recent evidence suggests that Ca^{2+} influx is also carrier-mediated and energy-requiring (Flik *et al.* 1985). Influx of trace metals such as Zn, however, might occur simply by passive entry down electrochemical gradients (Pentreath, 1973; Bryan, 1979) although this does not preclude carrier-mediation (Bryan, 1979). Zinc, at least in mammals, is tightly bound to plasma and intracellular ligands, reducing the internal free ion activity to very low levels (Hambidge *et al.* 1986). As well, the transepithelial electrical potential across the gills is generally negative inside in fresh water (see Potts, 1984), also favouring the passive entry of Zn. Actual measurements of Zn influx using the isolated, perfused head preparation, however, indicated that influx was not a simple linear function of waterborne [Zn] (Spry & Wood, 1988). Rather, influx was saturable, suggesting that uptake occurred *via* carrier mediation or through a saturable channel.

The first aim of the present study was simply to obtain reliable measurements of Zn influx from the water into the rainbow trout *in vivo*. There have been no previous determinations in freshwater fish. We therefore critically evaluated two existing flux techniques in common use for other electrolytes: disappearance of radiotracer (i.e. ^{65}Zn) from the water, or appearance in the fish by whole-body counting. Neither method proved satisfactory for measuring short-term Zn uptake. We therefore developed a new kinetic method based upon calibrating cannulated fish by infusion with ^{65}Zn at known rates. The calibration data were then applied to exposed fish from which only a single terminal blood sample was taken.

Our second goal was to apply this new method to determine the rate of Zn influx as a function of waterborne [Zn]. Based on our previous *in vitro* evidence (Spry & Wood, 1988), we hypothesized that part or all of the influx would saturate with increasing waterborne [Zn]. A component which did not saturate would suggest the presence of simple diffusive uptake.

Our final aim was to determine whether acute changes in waterborne $[Ca^{2+}]$ would cause changes in Zn influx, and the kinetic nature of any interaction which

Table 1. *Experiments performed*

	Waterborne $[Ca^{2+}]$ (mequiv l^{-1})	Waterborne [Zn] (mg l^{-1})	Fish mass (g)	*N*
I. Loss of Zn from the water	2	0·5	250–350	5
II. Whole-body uptake of Zn	2	0·5	2–4	60
III. Change in blood Zn activity during waterborne exposure	2	0·7	223–310	5
IV. Zn kinetics in blood				
bolus injection	2	background	180–325	7
constant rate infusion	2	background	194–346	6
V. Steady-state plasma Zn concentrations during constant-rate infusion	2	background	236–300	27
VI. Effect of waterborne $[Ca^{2+}]$ on Zn influx	see Table 3	see Table 3	273–341	168

occurred. There are several reasons to suspect that Ca^{2+} might interact with Zn. Waterborne $[Ca^{2+}]$ is an important determinant of branchial permeability (Eddy, 1975; McWilliams, 1983), and may also restrict access of transported electrolytes to their carriers or channels (McDonald, 1983). Furthermore, both Zn and Ca^{2+} are divalent cations and could conceivably compete for the same transport mechanism. We have demonstrated that waterborne Zn blocks Ca^{2+} uptake *in vivo* (Spry & Wood, 1985), so a reciprocal effect might be anticipated. Increased water hardness (mostly Ca^{2+} and Mg^{2+}) attenuates the acute toxicity of many trace metals, Zn in particular (Spear, 1981). Pagenkopf (1983) has hypothesized that the underlying mechanism is one of a competitive interaction between Ca^{2+} and toxic trace metals at the gill surface.

Materials and methods

Experimental animals

Underyearling rainbow trout, *Salmo gairdneri* (180–320 g), and fingerlings (2–4 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. They were held separately in flowing, charcoal-dechlorinated, Hamilton city tapwater ($[Na^+] = 0{\cdot}6$, $[Cl^-] = 0{\cdot}7$, $[K^+] = 0{\cdot}05$, $[Ca^{2+}] = 2{\cdot}0$, $[Mg^{2+}] = 0{\cdot}6$, titratable alkalinity = 1·98 mequiv l^{-1}, pH 8·1) at ambient temperature for several months before use and fed a pelleted, commercial diet (Martin Feed Mills, Elmira, Ontario). Prior to testing, trout were temperature-acclimated to 15 °C at an overall rate not exceeding 1 °C day^{-1}. The large trout were acclimated in batches of 10–20 in 500-l polyethylene tanks and not fed under these conditions; water was changed on alternate days. Small trout, owing to the smaller biomass, were temperature-acclimated in the holding tanks by control of water inflow rate. Some experiments (Table 1) assessed Zn influx at nominal waterborne $[Ca^{2+}]$ of 10, 5, 2 (tapwater), 1 and 0 mequiv l^{-1}. Water harder than tapwater was created by the addition of $CaCl_2$

Table 2. *Mean measured values of major waterborne ions during waterborne Zn exposures for experiment VI*

Nominal $[Ca^{2+}]$	Measured $[Ca^{2+}]$	$[Mg^{2+}]$	$[Na^{+}]$	$[K^{+}]$	$[Cl^{-}]$
10	9·71 ± 0·02 (36)	0·55 ± 0·02 (36)	0·61 ± 0·01 (36)	0·05 ± 0·02 (36)	8·38 ± 0·04 (18)
5	4·72 ± 0·01 (18)	0·60 ± 0·01 (18)	0·63 ± 0·03 (18)	0·05 ± 0·03 (18)	3·36 ± 0·04 (18)
2	2·00 ± 0·01 (108)	0·58 ± 0·00 (108)	0·64 ± 0·00 (108)	0·05 ± 0·02 (108)	0·71 ± 0·02 (54)
1	1·02 ± 0·02 (18)	0·60 ± 0·02 (18)	1·31 ± 0·03 (18)	0·09 ± 0·05 (18)	0·52 ± 0·02 (18)
0·5	0·05 ± 0·00 (42)	0·59 ± 0·01 (42)	2·23 ± 0·02 (42)	0·10 ± 0·04 (42)	0·48 ± 0·02 (21)

Values are means ± S.E. (*N*).
All ion concentrations are in mequiv l^{-1}.

which, of course, also raised $[Cl^{-}]$ above tapwater levels. Water softer than tapwater was prepared by adding to distilled water $NaHCO_3$, magnesium salt $[4MgCO_3.Mg(OH)_2.4H_2O]$, KCl and NaCl and, where necessary, $CaCl_2$. The water was vigorously aerated and the pH adjusted to 8·1 with NaOH prior to use. This duplicated tapwater concentrations for all measured variables (including titratable alkalinity) except Na^{+} and Ca^{2+}. Water samples for ionic analysis were routinely drawn throughout all experiments. Mean measured values are given in Table 2.

Experimental protocol

Flux measurements by disappearance of radioisotope from the water were performed on large trout, each in a clear acrylic box (approx. 1 l capacity) within a larger (approx. 14 l capacity) black acrylic box (McDonald, 1983). Boxes were held on a wet table over which chilled water flowed, to maintain an experimental temperature of 15 °C. Trout were acclimated to the boxes a minimum of 24 h prior to flux measurements. During this time, fresh tapwater flowed continuously through them. Perimeter aeration and an air-lift at the rear of the smaller box ensured adequate water circulation. The actual flux measurements were performed by stopping water flow into the larger box. Sufficient stock solution of Zn (as $ZnSO_4$) plus ^{65}Zn tracer (New England Nuclear) was added to give 0·5 mg Zn l^{-1} and a total of approx. 50 μCi of isotope. Water samples were taken prior to Zn addition (0 h) and at 0·5, 1, 2, 4, 6, 8, 10 and 12 h. A flux box containing water, but no fish, served as a control. Water samples were acidified (2 μl ml^{-1})

with high-purity HNO_3. Subsamples were counted for gamma activity in a well-type counter (Nuclear-Chicago model 1084), corrected for decay and counting geometry where necessary, or analysed for total zinc by atomic absorption spectrophotometry (Varian AA 1275).

To estimate influx based upon appearance of radioisotope in the fish by whole-body counting, 60 fingerlings were placed into a single 14 l flux box (no inner box) filled with tapwater ($Ca^{2+} = 2\,mequiv\,l^{-1}$). Six fish were sampled for control values, then Zn plus radiotracer was added as above. Six fish, plus water, were sampled at 0·25, 0·5, 1, 2, 4, 8, 12 and 27 h. Fish were dipped in flowing fresh water, pithed, blotted dry and whole bodies were counted for gamma activity in the well counter. Water samples were analysed as above.

For the development of the new kinetic flux measurement technique, large trout had indwelling cannulae implanted, either in the dorsal aorta alone (Soivio *et al.* 1972) or in the dorsal aorta and the caudal vein (Wilkes *et al.* 1981), under MS-222 anaesthesia. The fish were allowed to recover for 48 h prior to use, and the cannulae were periodically flushed with heparinized (50 i.u. ml^{-1}) Cortland saline (Wolf, 1963). To avoid contamination, injections or infusions of ^{65}Zn were performed *via* the caudal catheter, while blood was sampled *via* the dorsal aortic catheter. Blood samples (50–100 μl) for ^{65}Zn activity and total [Zn] were centrifuged at 8000 *g*, and the plasma counted or assayed as above for water.

In some experiments, $ZnSO_4$ plus ^{65}Zn tracer was added to the external tapwater ($[Zn] = 0{\cdot}7\,mg\,l^{-1}$) as in the previous flux experiments and fish exposed for 12 h. In others, a bolus of Zn plus ^{65}Zn sufficient to double the total plasma Zn was dissolved in 1 ml of Cortland saline, injected into the dorsal aorta, and flushed in with an additional 1 ml of saline. The experiments lasted 120 h. In alternate protocols, Zn plus ^{65}Zn in Cortland saline was infused into the caudal vein for 36 h at a constant rate of 15 $\mu l\,min^{-1}$ using a peristaltic pump (Gilson Minipulse). A variety of dosage rates was achieved by adjusting the concentration of Zn in the infusate, thus keeping the flow rate constant. In all these protocols, [Zn] and radioactivity were periodically measured in water and plasma samples.

In the perfected protocol, Zn influx was measured in uncannulated trout individually exposed to $ZnSO_4$ plus ^{65}Zn tracer in the external water (closed system, volume 14 l) for 24 h. Just prior to addition of Zn, the system was thoroughly flushed with water of the desired [Ca^{2+}]. Zn concentration and radioactivity were measured in single terminal plasma samples drawn by caudal puncture from anaesthetized fish (0·5 g MS-222 l^{-1}). Water [Zn] and ^{65}Zn activity were monitored several times during the exposure.

No mortality or morbidity occurred during any of these experiments. Other workers have reported acute toxicity at Zn concentrations less than 1·5 $mg\,l^{-1}$ when hardness was less than 1 $mequiv\,l^{-1}$ as Ca^{2+} (see Spear, 1981; Spry & Wood, 1985). However, fingerling trout flourished for 16 weeks at 0·59 $mg\,l^{-1}$ (Spry *et al.* 1988). At higher Zn concentrations the high alkalinity in our tests, even when hardness was low, probably prevented mortality by exerting an ameliorating effect distinct from that of Ca^{2+} (Holcombe & Andrew, 1978).

Calculations

The distribution volume or radiospace for ^{65}Zn (V_d in $ml\,kg^{-1}$) was estimated as:

$$V_d = \frac{Q^*_{int}}{\text{plasma radioactivity} \times W}, \quad (1)$$

where Q^*_{int} is the total radioisotope activity (counts min^{-1}) in the fish, W is the fish mass in kg, and plasma radioactivity is in counts $min^{-1}\,ml^{-1}$.

Traditional flux calculations were based upon the disappearance from the water of ^{65}Zn of known specific activity, on the assumption that the system was composed of only two compartments. Thus, Zn which left the water entered the fish. Since back-flux of isotope did not occur in the short term, as shown by later infusion experiments, a simplified case of the general flux equation (Kirschner, 1970) was used:

$$J_{in} = \frac{Q_{out} \times (\ln Q^*_{out(0)} - \ln Q^*_{out(t)})}{t \times W}, \quad (2)$$

where J_{in} is Zn influx rate (nequiv $kg^{-1}\,h^{-1}$), Q_{out} is the total mass of element in the medium, Q^*_{out} is the total isotope activity (counts min^{-1}) in the medium and t is the elapsed time (h).

For small trout, the total Zn accumulated with time was calculated from the activity of the fish and the mean specific activity of the water sample during the period. Actual flux rates were then calculated by dividing by the elapsed exposure time. The same approach was used in measuring uptake rates in the short-term experiments with large trout which were designed to factor out the sites of adsorption. Here, however, activity of the whole fish was determined from homogenate counts rather than whole-body counts.

In the development of the kinetic technique for flux measurements, two models were used to fit equations to data. The first model was applied to the plasma radioactivity *versus* time and is an exponential saturation:

$$y = C_{ss}(1-e^{-kt}), \quad (3)$$

where y is the plasma radioactivity (counts $min^{-1}\,ml^{-1}$), C_{ss} is the steady-state activity in the plasma, k is the rate constant, and t is the time. The rising section of the curve represents the addition of labelled Zn to the unlabelled pool already present in the plasma, the influx of labelled Zn occurring from (i) uptake from the water in the case of the waterborne exposure, or (ii) inflow *via* the caudal cannula in the case of the infusion. Steady state is reached when the amount of label (fractional removal of the total) cleared from the plasma equals the amount entering from either the water or the infusion (see Shipley & Clark, 1972).

The second model was used to describe the relationship between measured Zn influx rate and the concentration of waterborne [Zn]. Since the latter is essentially the substrate concentration, and since a saturable component was observed (see

Results), the model is based upon enzyme kinetics, and uses a hyperbolic saturation (Michaelis–Menten) equation:

$$J_{in} = \frac{J_{max}[Zn]}{K_m + [Zn]}, \tag{4}$$

where J_{in} is the influx rate (nequiv $kg^{-1} h^{-1}$), [Zn] is the waterborne substrate concentration (μequiv ml^{-1}), J_{max} is the maximum flux rate and K_m is the [Zn] for half-maximum flux rate. In cases where there appeared to be an additional nonsaturable component, an alternative model, which included a linear transport term was fitted to the data:

$$J_{in} = \frac{J_{max}[Zn]}{K_m + [Zn]} + m[Zn], \tag{5}$$

where m is the slope of the linear component which passes through the origin. Values for K_m and V_{max} (and m, where used) were determined by iterative nonlinear curve fitting (Johnston, 1985). Starting values for the iteration were those determined by eye from plots of the data. Goodness of fit was assessed from the residual sum of squares.

In analysing the influence of waterborne [Ca^{2+}] on Zn influx, the possibility of competitive inhibition was considered. In such a situation, the true K_m for Zn influx would be related to the apparent K_m in the presence of an inhibitor by the relationship:

$$K_m = \frac{\text{apparent } K_m}{1 + ([I]/K_i)}, \tag{6}$$

where [I] is the inhibitor (Ca^{2+}) concentration and K_i is the inhibitor constant (Segel, 1976). All data are expressed as means ± S.E. (N).

Results

Flux rate calculations using traditional techniques

Attempts to calculate Zn influx rates in large trout based upon disappearance of isotope from the water revealed significant adsorption of both stable and radiotracer Zn to the flux boxes. Over the time course of the experiment, both the activity (Fig. 1) and the total [Zn] in the water fell by between 7 and 30%, such that the specific activity remained relatively constant. This was true for both the control box and the ones which contained trout. This result clearly showed that the method was insensitive to the presence of the fish. Calculation of flux rates (Fig. 2), *although clearly inappropriate*, demonstrated peak rates of about 60 000 nequiv $kg^{-1} h^{-1}$ which fell over the first 4 h and stabilized at about 8000 nequiv $kg^{-1} h^{-1}$.

Results with fingerling trout, based upon whole-body counting, however, clearly showed that fish accumulated the isotope from the water. The amount accumulated continued to increase linearly with time (Fig. 3). Calculation of the influx rate, however (Fig. 4), indicated initial rapid uptake, about 6000 nequiv $kg^{-1} h^{-1}$

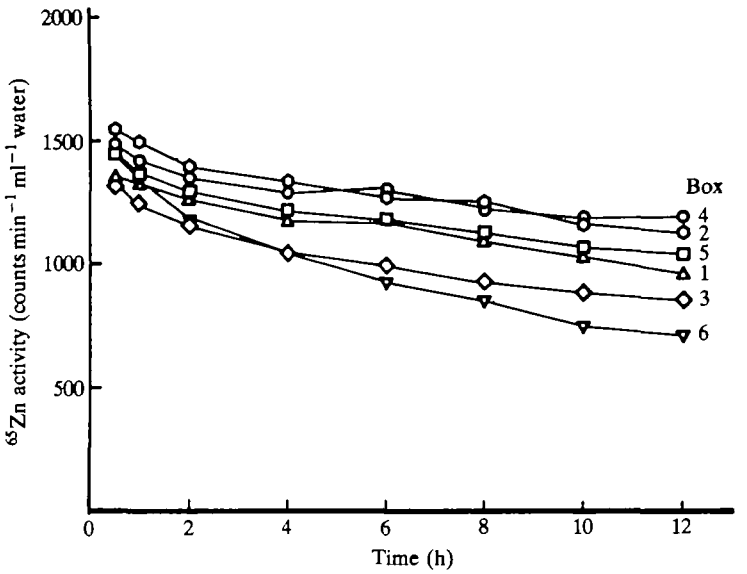

Fig. 1. Disappearance of ^{65}Zn activity from tapwater. Each of five flux boxes contained 14 l of tapwater and one 250–350 g trout. Box 3 held only water. Waterborne [Zn] = 0·5 mg l^{-1} = 15 μequiv l^{-1}, [Ca^{2+}] = 2·0 mequiv l^{-1}.

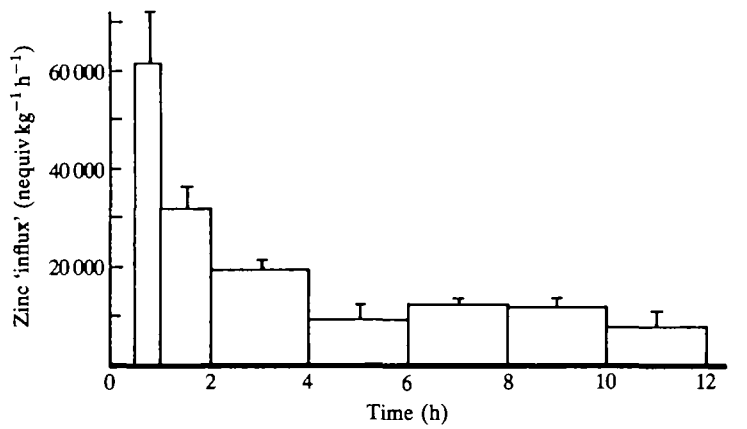

Fig. 2. Zinc 'influx' based upon disappearance of counts from tapwater, mean ± S.E. (N = 6). Details as in Fig. 1.

during the first 15 min of exposure. Thereafter, uptake fell exponentially, to about 700 nequiv kg^{-1} h^{-1} by 27 h. This rapid initial uptake undoubtedly represented adsorption to the exterior of the fish with little internalization of Zn.

These two types of experiments, which attempted to measure influx using a simple two-compartment model, indicated the presence of additional compartments which corresponded to adsorption to the flux box in the first instance, and to

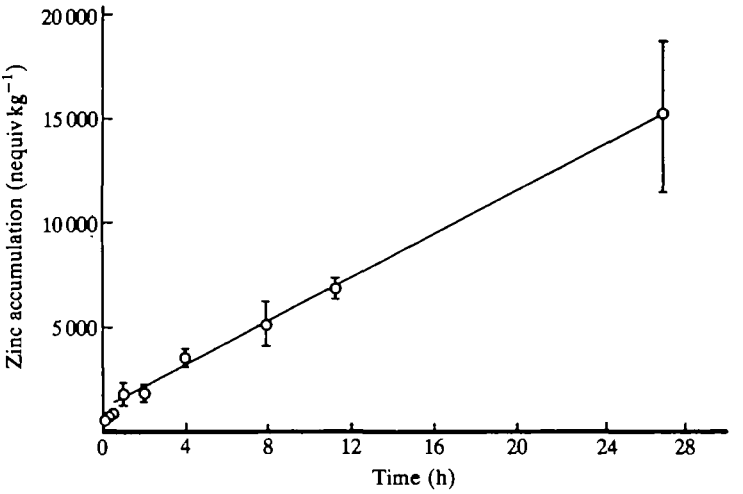

Fig. 3. Net Zn accumulation based on ^{65}Zn accumulation by fingerling (2–4 g) rainbow trout, mean ± S.E. ($N = 6$). Waterborne [Zn] = 0·5 mg l^{-1} = 15 μequiv l^{-1}, [Ca^{2+}] = 2·0 mequiv l^{-1}.

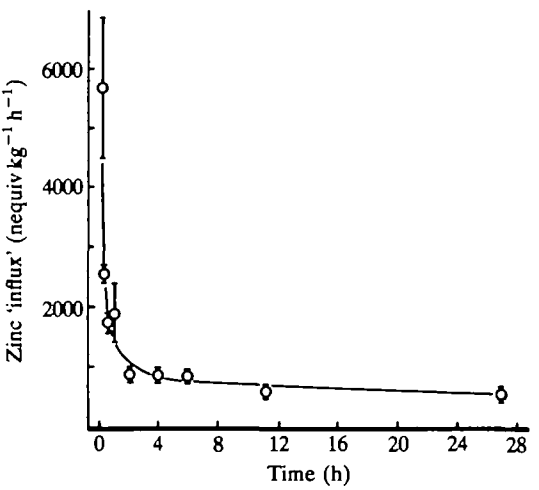

Fig. 4. Zinc 'influx' in fingerling trout based upon whole-body ^{65}Zn accumulation in tapwater. Details as in Fig. 3.

the exterior of the fish in the second. The presence of these compartments clearly resulted in erroneous estimates of influx rates, especially over the first few hours of exposure.

Flux rate calculations based upon blood sampling

To circumvent these difficulties, sampling of an internal tissue containing only

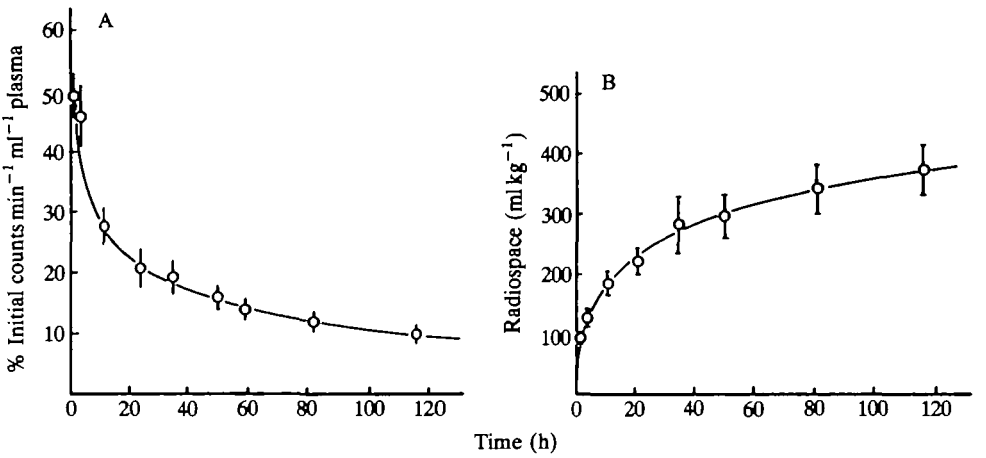

Fig. 5. (A) Clearance of a bolus of radiolabelled Zn sufficient to double the total-plasma [Zn], from the plasma of rainbow trout in tapwater ($[Ca^{2+}] = 2{\cdot}0$ mequiv l^{-1}), mean ± S.E. ($N = 7$). (B) Change in the radiospace of Zn after infusion of a bolus of radiolabelled Zn sufficient to double the total plasma [Zn].

absorbed Zn was attempted. Blood is a tissue particularly suited to repeated sampling, and therefore a number of tests were performed to determine the suitability of influx determinations using blood samples. These assessed the characteristics of ^{65}Zn clearance from the blood, whether ^{65}Zn from the water entered the blood space in sufficient quantities to be measured, and the quantitative relationships between ^{65}Zn entry rate and plasma concentrations.

If ^{65}Zn enters the blood space and is completely retained there, at least in the short term, then influx could simply be estimated from the product of plasma radioactivity and plasma volume, factored by the appropriate water specific activity and time. However, if ^{65}Zn is cleared from the plasma at a significant rate, then the analysis becomes more complex. To evaluate this situation, large trout fitted with indwelling catheters were given a bolus of Zn plus ^{65}Zn sufficient to double total plasma [Zn]. Blood samples were taken over 120 h and plasma recovered and counted for radioactivity. Zn was rapidly lost from the plasma in typical first-order fashion (Fig. 5A). In fact, after 1 h, only about 50% of the infused dose was retained. Collection of water samples over the experiment revealed that excretion of the dose was minimal (i.e. <1%). The unlikely possibility that Zn was lost from the fish, but not measured due to the adsorption onto the box, was discussed by Spry & Wood (1988). First, branchial zinc loss from the blood is not favoured by either electrical or chemical gradients. This is due to a transepithelial potential which is negative inside in fresh water, and a blood free [Zn] which is vanishingly small owing to binding by blood proteins. Second, urinary excretion is also very low even in Zn-exposed trout (Spry & Wood, 1985). Zn lost from the plasma was thus clearly redistributed to other tissues and, in fact, the radiospace (equation 1) for Zn (when plasma [Zn] was doubled) increased

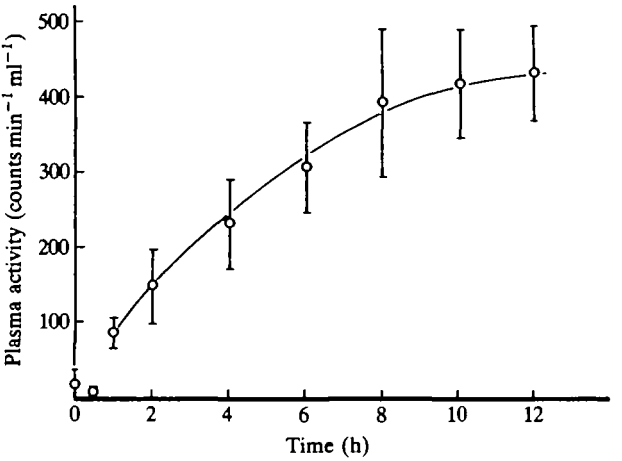

Fig. 6. Appearance of radiolabelled Zn activity in the plasma of large rainbow trout exposed to radiolabelled Zn in tapwater ([Zn] = 0·7 mg l^{-1}, [Ca^{2+}] = 2·0 mequiv l^{-1}), mean ± S.E. (N = 5).

from about 100 ml kg^{-1} (approximately double the blood volume; Milligan & Wood, 1982) at 1·5 h after injection to about 400 ml kg^{-1} after 120 h (Fig. 5B). This loss from the plasma and changing distribution volume clearly invalidated an approach based simply on the product of radioactivity and distribution volume. Nevertheless, if ^{65}Zn were to enter from the water in measurable quantities and follow predictable patterns in the plasma, then a kinetic analysis would still be feasible.

To assess this possibility, cannulated trout were exposed to waterborne Zn plus ^{65}Zn as described above, and sampled at various periods over 12 h. Plasma radioactivity rose gradually, approaching steady-state values by 12 h (Fig. 6). There appeared to be a time lag of 0·5–1 h before measurable activity appeared in the plasma. Nevertheless, the data were well-described by the exponential saturation equation (equation 3). Nonlinear regression through the means gave C_{ss} = 7·1 ± 0·4 nequiv ml^{-1} and k = 0·18 h^{-1}. Since there are undoubtedly multiple exits from the plasma to the tissues, each with a characteristic rate constant, and since these constants are additive, k in this case is the lumped rate constant. Biological half-life (ln2/k) for plasma Zn in this case was 3·8 h. Since 5–7 half-lives are required to reach a plateau, true steady-state concentrations would be reached in 19–27 h. This experiment clearly demonstrated that Zn entered the plasma in measurable quantities and followed a predictable time course of accumulation in the blood plasma, eventually reaching equilibrium.

If the pool size were accurately known, then influx would be given by the product of the rate constant and pool mass. However, the pool size was not simply the mass of Zn in the plasma, since plasma specific activity, despite closely approaching steady-state value, was still <3 % of that in the water. This indicated

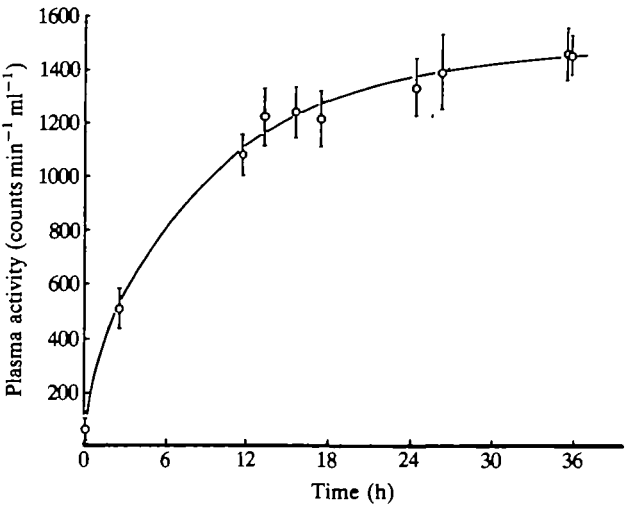

Fig. 7. Appearance of radiolabelled Zn over time, in the plasma of rainbow trout infused at a single constant rate (174 ± 13 nequiv kg^{-1} h^{-1}), in tapwater ([Ca^{2+}] = 2·0 mequiv l^{-1}), mean ± S.E. ($N = 6$).

either a very large endogenous turnover of plasma Zn (pool size much greater than total plasma Zn) or a large compartment of non-exchangeable plasma Zn.

To circumvent the difficulty of the unknown pool size (in terms of both mass and volume), an empirical approach based on C_{ss} was taken, in which large fish were 'calibrated' by infusion of Zn plus ^{65}Zn over a range of possible influx rates. This infusion was analogous to the entry of Zn into the plasma across the gill. A typical infusion curve of plasma activity *vs* time (Fig. 7) indicated that steady-state values were reached by 24–36 h, in agreement with the previous analysis. Infusions at several lower doses (not shown) confirmed that true plateaus were achieved by 24–36 h. As with bolus injections, measured losses of ^{65}Zn to the water were negligible (<1 % of infused dose).

A scatterplot of C_{ss} *vs* the infusion rate of exogenous Zn into the plasma (J_{in}) of 27 fish revealed a linear relationship over a wide range of influx rates (Fig. 8, $J_{in} = 8{\cdot}43C_{ss} + 4{\cdot}94$, $r = 0{\cdot}88$, $P < 0{\cdot}01$). Using this relationship, it was then possible to calculate actual influx based upon a single terminal blood sample from uncannulated trout after 24–36 h of exposure to waterborne Zn.

To verify that this relationship remained valid under different environmental conditions (e.g. very low water [Ca^{2+}]) where Zn flux rates might be higher, additional infusions were performed at higher 'influx rates', and also under the lowest water [Ca^{2+}] (0·05 mequiv l^{-1}). These rates were not significantly different from the values predicted from C_{ss} by the original relationship (paired *t*-test, $P < 0{\cdot}2$), indicating that the regression could be extrapolated beyond the original calibration range, and also applied in water of very different quality.

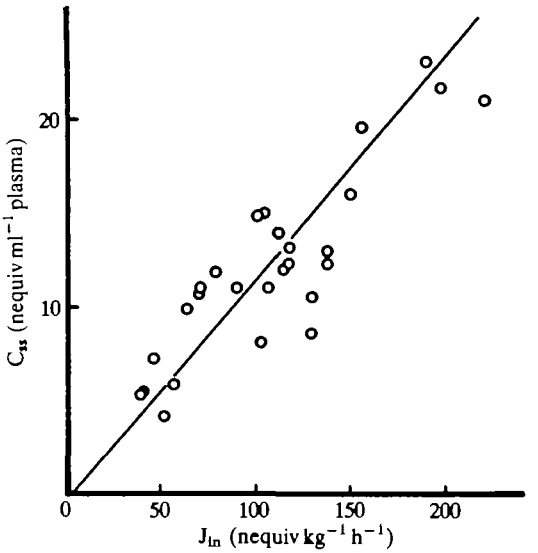

Fig. 8. Steady-state concentrations (C_{ss}) of exogenous Zn in the plasma of 27 trout at various constant-rate infusions of radiolabelled Zn. All data from trout in tapwater ($[Ca^{2+}] = 2{\cdot}0$ mequiv l^{-1}).

The kinetics of Zn influx and the influence of Ca^{2+}

When uncannulated large trout were exposed to a range of waterborne [Zn] (at constant specific activity) in tapwater ($Ca^{2+} = 2{\cdot}0$ mequiv l^{-1}), and Zn influx calculated from a terminal plasma sample, influx did not show a simple linear increase with concentration. Instead, influx rose rapidly and then saturated above a waterborne [Zn] of 0·4 mg l^{-1} (Fig. 9). The data were well-described by the Michaelis–Menten model (equation 4), suggesting either mediated transport or entry through a selective pore. Attempts to fit a transfer model which added a linear term (equation 5) were unsuccessful. The J_{max} was 314 nequiv kg^{-1} h^{-1}, and the apparent K_m was 0·24 mg l^{-1} (7·3 μequiv l^{-1}; Table 3). This value for J_{max}, which represents true transbranchial transfer, was considerably lower (2- to 25-fold) than that given by traditional (and erroneous) indirect methods such as loss of tracer from the water compartment (Fig. 2) or whole-body uptake (Fig. 4).

The effect of acute changes in waterborne $[Ca^{2+}]$ on the relationship between influx rate and waterborne [Zn] was studied using water with $[Ca^{2+}] = 9{\cdot}7$, 4·7, 1·02 and 0·05 mequiv l^{-1}. Two of these experiments used water harder than tapwater (2·0 mequiv l^{-1}), and two softer. Increases in waterborne $[Ca^{2+}]$ to 4·7 or 9·7 mequiv l^{-1} had only a small effect on the J_{max} (Fig. 10, Table 3). They did, however, greatly decrease the rate of influx at lower [Zn], and thus increase the apparent K_m by a factor of 6. Individual variability also decreased as $[Ca^{2+}]$ increased. When the fitted curves were plotted as double reciprocal plots, acute addition of Ca^{2+} clearly acted as a competitive inhibitor (Fig. 11A).

Acute removal of Ca^{2+} increased influx at all Zn concentrations. Variability of

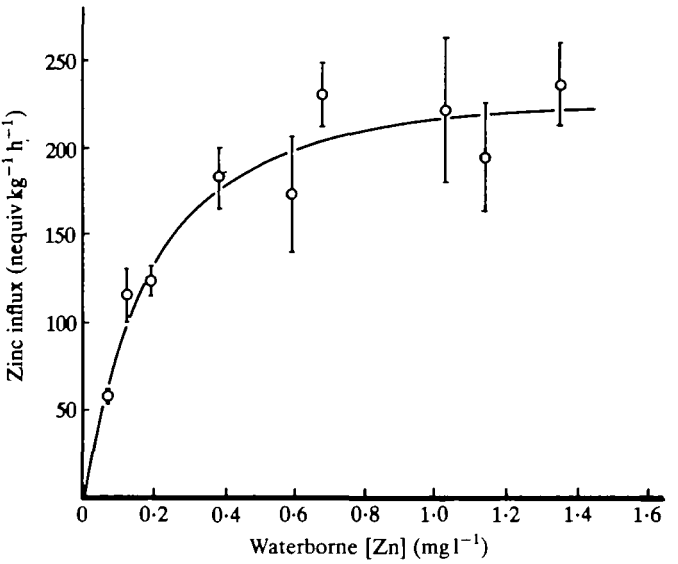

Fig. 9. Zinc influx into 54 large rainbow trout at various waterborne Zn concentrations in tapwater ($[Ca^{2+}] = 2{\cdot}0$ mequiv l^{-1}), based upon the steady-state plasma concentrations of radiolabelled Zn (C_{ss}), mean ± S.E. ($N = 6$) for each point.

Table 3. *J_{max} and apparent K_m for Zn influx into rainbow trout at various waterborne calcium concentrations*

$[Ca^{2+}]$ (mequiv l^{-1})	J_{max} (nequiv $kg^{-1} h^{-1}$)	Apparent K_m		Linear term ($l\,kg^{-1} h^{-1}$)
		(mg l^{-1})	(μequiv l^{-1})	
9·7	458	1·53	46·8	–
4·7	364	0·85	26·0	–
2·0	314	0·24	7·3	–
1·0	277	0·12	3·7	178

the data also increased. At $[Ca^{2+}] = 1{\cdot}02$ mequiv l^{-1}, influx appeared to saturate above 1·0 mg Zn l^{-1}. These data fitted the model (equation 5) containing an unsaturable component much better than they did the simpler model (equation 4). The J_{max} and apparent K_m of the saturable component, when plotted as a double reciprocal, compared well with the trends of data from experiments with higher waterborne $[Ca^{2+}]$ (Fig. 11A, Table 3). The apparent K_m was lowered to 0·12 mg l^{-1} (3·7 μequiv l^{-1}). This, again, suggests that Ca^{2+} acts as a competitive inhibitor of Zn influx. At the lowest $[Ca^{2+}]$, however, influx increased almost linearly at $[Zn] \geq 0{\cdot}5$ mg l^{-1}, with no apparent saturation. The data did not fit either model, but could well reflect the presence of a dominant unsaturable component masking the saturable component. It was clear that acute removal of

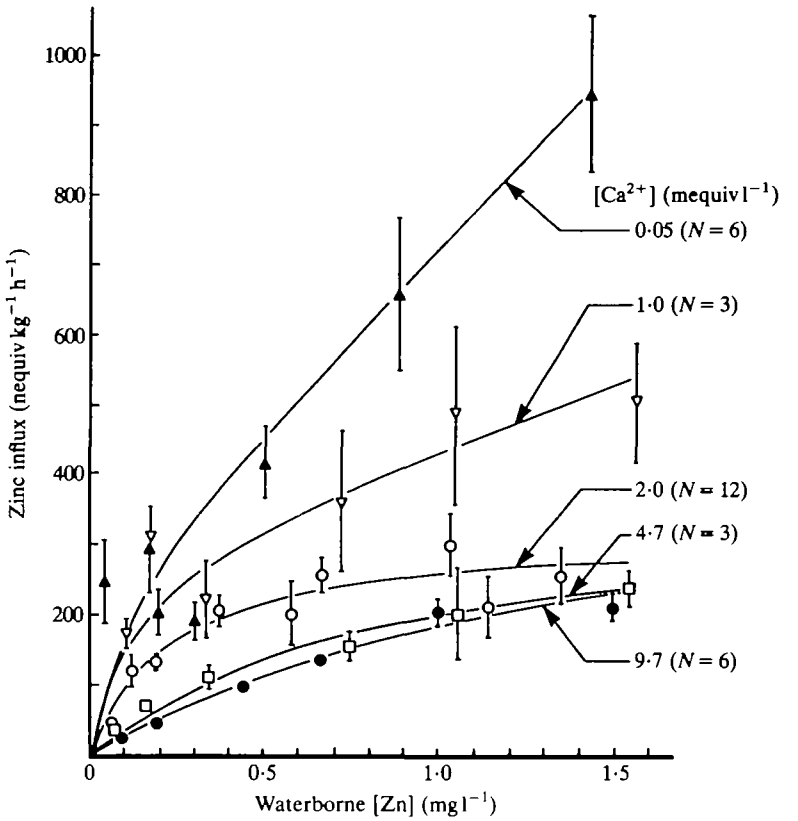

Fig. 10. Zinc influx into 168 large rainbow trout at various waterborne Zn concentrations in water at five different $[Ca^{2+}]$. Rates are based upon the steady-state Zn radioactivity in the plasma (C_{ss}), mean ± S.E.; N is given in parentheses for the average number of fish for each point on a curve. Curves were generated using equation 4 for $[Ca^{2+}] = 9{\cdot}7$, 4·7 and 2·0 mequiv l^{-1} and equation 5 for $[Ca^{2+}] = 1{\cdot}0$ mequiv l^{-1}, using data in Table 3. Curve for $[Ca^{2+}] = 0{\cdot}05$ mequiv l^{-1} was fitted by eye.

most of the waterborne Ca^{2+} dramatically increased the transfer of Zn across the gill.

Since Ca^{2+} inhibition of Zn influx resembled competitive inhibition, it was possible to determine an inhibitor constant (K_i) from a plot of apparent K_m/V_{max} *vs* $[Ca^{2+}]$ (Fig. 11B; Segel, 1976). This gave a $K_{i,Ca}$ of 0·48 mequiv l^{-1}. Interestingly, this is very similar to the K_m for Ca^{2+} uptake (280 ± 70 μequiv l^{-1}) determined by Perry & Wood (1985) for similarly acclimated trout. Since all the influx measurements were performed with some Ca^{2+} present in the water, the apparent K_m values were all determined in the presence of an inhibitor. The mean value for the true K_m (equation 6) over waterborne Ca^{2+} concentrations from 1·02 to 9·7 mequiv l^{-1} was 1·0 ± 0·1 μequiv l^{-1} (0·06 mg l^{-1}) ($N = 4$). The affinity of the uptake system therefore appears to be nearly 300-fold higher for Zn than for Ca^{2+}.

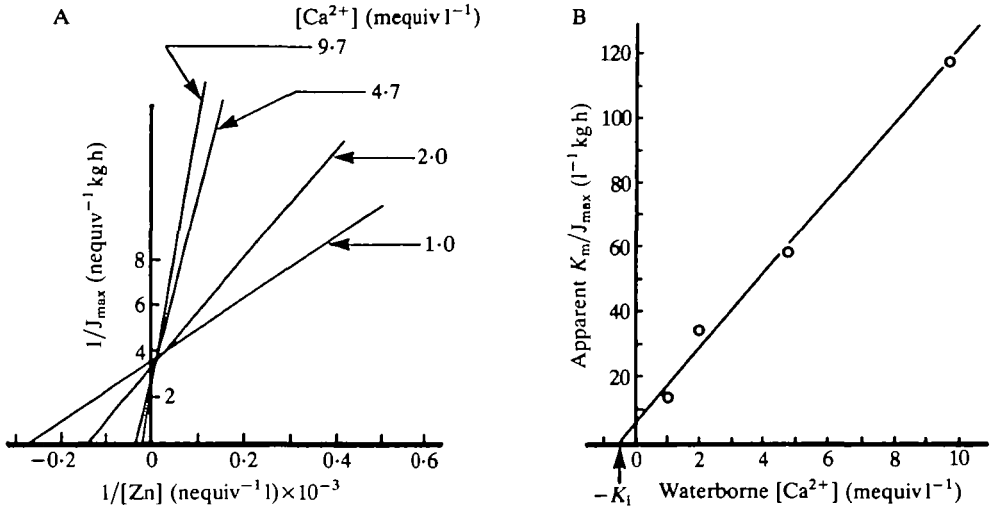

Fig. 11. (A) Double reciprocal plot of data from Fig. 10, to show the nature of the competitive interaction between Zn and Ca^{2+}. (B) Determination of the inhibitor constant for Ca^{2+}. The ratio of apparent K_m to J_{max} is plotted against $[Ca^{2+}]$, and the x-intercept = $-K_i$. The regression equation was $y = 11{\cdot}6x + 5{\cdot}6$; $r = 0{\cdot}996$.

Discussion

Traditional methods of partitioning unidirectional fluxes in fish, of bulk ions such as Na^+ and Cl^- (see Kirschner, 1970) and K^+ (Eddy, 1985), have successfully used a two-compartment, closed model with fish and water being the only compartments. Implicit in this model is the assumption that isotope which disappeared from the water entered the organism, with little or no adsorption. Adsorption was a problem with whole-body Ca^{2+} flux (Hõbe *et al.* 1984), although this difficulty was largely eliminated by rinsing in solutions of high unlabelled $[Ca^{2+}]$ (Perry & Wood, 1985). In the present study, significant adsorption also occurred with Zn, which could not be removed by rinsing in high [Zn] solutions (unpublished results). Zn has a high affinity for available ligands, and thus is not readily removed. Moreover, the true influx rate is low compared with all the bulk ions, and adsorption becomes a significant complication in short-term experiments. Estimation of influx based upon disappearance from the water was clearly invalid. These rates were up to two orders of magnitude higher than those derived from plasma measurements, as a result of Zn adsorption to both the fish and the flux box.

Attempts to measure influx using whole-body counting revealed that adsorption to the fish occurred very rapidly, resulting in rates of influx which were highest for the shortest exposure periods. With increasing exposure time, the adsorbed component was a smaller fraction of the total Zn uptake, and apparent flux rates declined, although they were still high compared with estimates using plasma activity. Some of the Zn which adsorbed to the integument was probably labile,

since Joyner (1960) found that brown bullheads (*Ictalurus nebulosus*) exposed to 6·0 mg Zn l^{-1} for 96 h lost 43 % of their accumulated ^{65}Zn after 1 day in clean water. In contrast, physically absorbed Zn was excreted very slowly, resulting in a half-life exceeding 250 days (Pentreath, 1973; Willis & Jones, 1977). Given this slow rate of loss for incorporated Zn, estimates of influx based upon whole-body uptake would undoubtedly be accurate given a sufficiently long exposure. However, such exposure would provide ample time for acclimatory changes in the gill which, although interesting in themselves, would preclude study of acute responses.

The lowest estimates of influx were those based upon applying the calibration curve to the activity of terminal plasma samples taken when steady-state activity was assured. Since these were also free from the confounding effect of adsorption of Zn to the exterior of the fish, they represent the best estimates of Zn influx presently available *in vivo* for trout. Previous attempts to measure the actual influx using an isolated, perfused head preparation (IPHP) yielded estimates about 30-fold lower than the present study at the same waterborne [Zn] (Spry & Wood, 1988). Reasons for this difference are unclear, but it seems likely that the IPHP underestimated the actual influx. Cd^{2+} influx *in vitro* using a similar IPHP was close to *in vitro* influx for Zn (Pärt & Svanberg, 1981). If the relationship that appears to exist for Zn also holds for Cd^{2+}, then influx measurements for Cd^{2+} *in vivo* may be greater than these authors have reported.

The final flux protocol which we have developed offers several benefits. First, it measures only Zn which enters the fish. In this regard it is similar to the IPHP. It has considerable advantage over the perfused head, however, in that once the calibration curve has been established, operative techniques are not required. Once steady-state plasma activity is assured, only a single terminal blood sample is used to estimate influx. Although the perfused head retains some advantage where the fluxing ion rapidly comes into isotopic steady state (e.g. Na^+, Cl^- and even Ca^{2+}), in that a range of concentrations can be tested in a single preparation, this was clearly not the case for Zn (Spry & Wood, 1988). As well, *in vivo* flux measurements are inherently more desirable than those *in vitro*, since responses by the fish, particularly those which are hormonally mediated, are not compromised. The present technique therefore shows particular promise for trace metals whose waterborne concentrations are low, and which show a strong tendency towards adsorption.

The kinetics of Zn uptake and the effects of Ca^{2+}

Environmental levels of Zn are rarely as high as the highest levels used in this study, some of which would probably be toxic given longer exposures, especially in water of low [Ca^{2+}] (Spry & Wood, 1985). Such high Zn concentrations were necessary, however, to define the shape of the curves. Regardless of this, the present data demonstrate clearly that at lower, more realistic values, small changes in [Zn] greatly increase Zn transfer.

Good influx measurements for trace metals are rare, and those that have

examined the response to graded concentrations even rarer. Saturability has now been demonstrated for Zn influx using both *in vivo* (present study) and *in vitro* methods (Spry & Wood, 1988). The relationship also appears to hold for Cd^{2+} using the IPHP (Pärt & Svanberg, 1981). This phenomenon implies a rate-limiting carrier or pore.

Changes in waterborne [Ca^{2+}] resulted in two rather different responses. Addition of Ca^{2+} to the water resulted in clear competitive inhibition of zinc transfer (Figs 10, 11). This was seen from the similarity in J_{max} (though see below), but with increasing apparent K_m as [Ca^{2+}] increased. The effects of Ca^{2+} removal were less clear, but again indicative of a competitive effect (Fig. 11B). Thus removal of Ca^{2+}, at least to 1·02 mequiv l^{-1}, acted on the saturable component as might be expected for the removal of inhibitor (similar J_{max}, lower apparent K_m).

However, at both levels of reduced [Ca^{2+}] there was an additional unsaturable component, which increased as the [Ca^{2+}] decreased. McWilliams (1983) postulated two types of Ca^{2+} sites on the gills of brown trout. The higher-affinity site was suggested to be the Ca^{2+} transport site, and the lower-affinity site may control permeability. Although the presence of a transport site on the apical surface is at variance with the recent hypothesis of Flik *et al.* (1985), the high-affinity site may still be related to active Ca^{2+} transport. Acute removal of Ca^{2+} from the water would thus have three effects: (i) removal of Ca^{2+} from some component of its transport process across the gill, allowing Zn to traverse the gill by this mechanism with less competition from Ca^{2+}; (ii) an increase in the general permeability of the gill, to permit entry of Zn across the gill by a nonspecific and unsaturable route, perhaps simple diffusion through paracellular channels; (iii) removal of Ca^{2+} with time, permitting toxic effects of Zn itself to further weaken the integrity of the paracellular channels, resulting in increased influx. This, in itself, might account for the absence of a plateau under these conditions and the observation that at the lowest [Ca^{2+}] the data were quite variable and did not fit any of the transport models used. The effects of Ca^{2+} removal are clearly complex.

The kinetic analysis of the Ca^{2+} *vs* Zn interaction indicated that the true K_m was 1·0 μequiv l^{-1}, and the K_i for Ca^{2+} as a competitive inhibitor of Zn influx was about 480 μequiv l^{-1}. Interestingly, the latter is very close to the measured K_m for branchial Ca^{2+} flux (280 ± 70 μequiv l^{-1}; Perry & Wood, 1985) for similarly acclimated rainbow trout. This further supports the case for competitive interaction between these two divalent cations, and suggests that the competition may be for the active transport process. The system appears to have 200- to 300-fold higher affinity for Zn than for Ca^{2+}.

Not only did waterborne Ca^{2+} act as an inhibitor of Zn influx, but the opposite effect, inhibition of net Ca^{2+} influx by acute exposure to Zn, has been seen in rainbow trout in natural soft water (Spry & Wood, 1985). A resultant fall in plasma [Ca^{2+}] was also noted. These data further support Ca^{2+}/Zn interaction in the gill. In that study, waterborne [Zn] was 24 μequiv l^{-1} and [Ca^{2+}] was 170 μequiv l^{-1}. In the light of the differences in affinity noted above, it is not surprising that this low level of [Zn] was sufficient to abolish Ca^{2+} uptake. Longer term exposure to Zn,

however, may permit acclimation and renewed Ca^{2+} uptake in the continued presence of the stressor, since a 16 week exposure to a [Zn] as high as 16 μequiv l^{-1} (0·53 mg l^{-1}) did not significantly alter plasma or whole-body [Ca^{2+}] in small trout (Spry *et al.* 1988).

Based upon the foregoing, we propose the following model. In the tapwater-acclimated and tested trout ([Ca^{2+}] = 2·0 mequiv l^{-1}), Zn influx is transcellular, probably *via* the same mechanism through the chloride cells as Ca^{2+} influx (Flik *et al.* 1985; Perry & Wood, 1985). Zn will passively enter the cell down its electrochemical gradient, perhaps through an aqueous pore, and then move out into the plasma through the basolateral border. The rate-limiting step could be at either the apical or the basolateral border. This latter step may involve active transport *via* the high-affinity Ca^{2+}-ATPase isolated by Flik *et al.* (1985). Interestingly, Ca^{2+}-ATPases in trout gill tissue *in vitro* were activated by Zn (Watson & Beamish, 1981), though this did not occur under all assay conditions, and it is not clear whether these were the same high-specificity Ca^{2+}-ATPases as those identified by Flik *et al.* (1985).

Over most of the range of [Ca^{2+}] studied, Ca^{2+} acts as a competitive inhibitor of Zn influx, either by titrating anionic groups on a pore or by binding to a carrier, thus requiring higher Zn concentrations to achieve the same uptake rate. As Ca^{2+} is removed, a second phenomenon, unsaturable transport, is superimposed upon this. This is almost certainly the result of changes in permeability, which are probably paracellular, though a transcellular route cannot be ruled out.

Numerous uncertainties remain. The available evidence certainly does not prove that Zn entering *via* the saturable route is necessarily transported actively. If we assume that 'free' Zn in the water is a large portion of the total (Campbell & Stokes, 1985), and that the 'free' Zn in the blood is vanishingly small, and given the small negative-inside transepithelial potential (McWilliams & Potts, 1978; Perry & Wood, 1985), then the driving force for Zn movement is clearly into the fish over most of the range of waterborne Zn concentrations tested. However, nothing is known about the gradient between the inside of the gill cell and the blood. Earlier studies with fish liver slices indicated that Zn was accumulated in a first-order fashion against a sevenfold gradient and that energy was not required (Saltman & Boroughs, 1960). In contrast, more recent work has documented an energy requirement for Zn uptake by cultured rat hepatocytes (Failla & Cousins, 1978) and rat intestinal segments (Kowarski *et al.* 1974).

The nominal 'purpose' of the Zn transport system also remains problematical. Is it a design feature of the gill, or just an 'accidental' effect of the necessary presence of the Ca^{2+} transport system? It could be argued since the apparent K_m for Zn uptake in normal tapwater (7·3 μequiv l^{-1}; Table 3) is rather high, almost into the toxic range (Spear, 1981) which would seldom be encountered by fish in the wild, that the system is not designed to transport Zn. However, this is misleading, for the apparent K_m is elevated in the presence of Ca^{2+}; the true K_m is much lower (1·0 μequiv l^{-1}). Certainly Zn uptake from the water occurs and is capable of preventing Zn deficiency in rainbow trout when dietary Zn is lacking (Spry *et al.*

1988). The much lower J_{max} for Zn uptake (approx. 400 nequiv $kg^{-1} h^{-1}$; Table 3) than for Ca^{2+} uptake (approx. 12 000 nequiv $kg^{-1} h^{-1}$; Perry & Wood, 1985) also suggests a design feature of the system. Thus, even though affinity is very high to enable uptake from low waterborne concentrations, the efficacy of Zn transport is very low, preventing high uptake rates which could be toxic.

Another uncertainty is the rather curious effect of $[Ca^{2+}]$ on the J_{max} for Zn influx. Instead of staying absolutely constant at varying $[Ca^{2+}]$, as expected for true competitive interaction, J_{max} showed a small but definite trend to increase as $[Ca^{2+}]$ increased (Fig. 11, Table 3). The meaning of this is unknown, for most standard complications (e.g. a contribution by noncompetitive inhibition) would tend to lower J_{max}. Perhaps high transport rates of Ca^{2+} in some way accelerate the movement of Zn, thereby slightly increasing the efficacy of transport.

Finally, chronic exposure to Zn may result in increased or decreased Zn transfer. The gill is very adaptable, particularly regarding the chloride cell composition (Laurent *et al.* 1985; Perry & Wood, 1985). Chloride cell proliferation would presumably increase Zn transfer, since increased chloride cell number and size were associated with increased Zn transfer across a perfused head preparation (Spry & Wood, 1988). Alternatively, other adaptations such as hormonally mediated changes in gill permeability might decrease transfer during chronic exposure. This is an important area for future investigation.

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